Furin inhibition prevents hypoxic and TGF -mediated blood-brain barrier disruption

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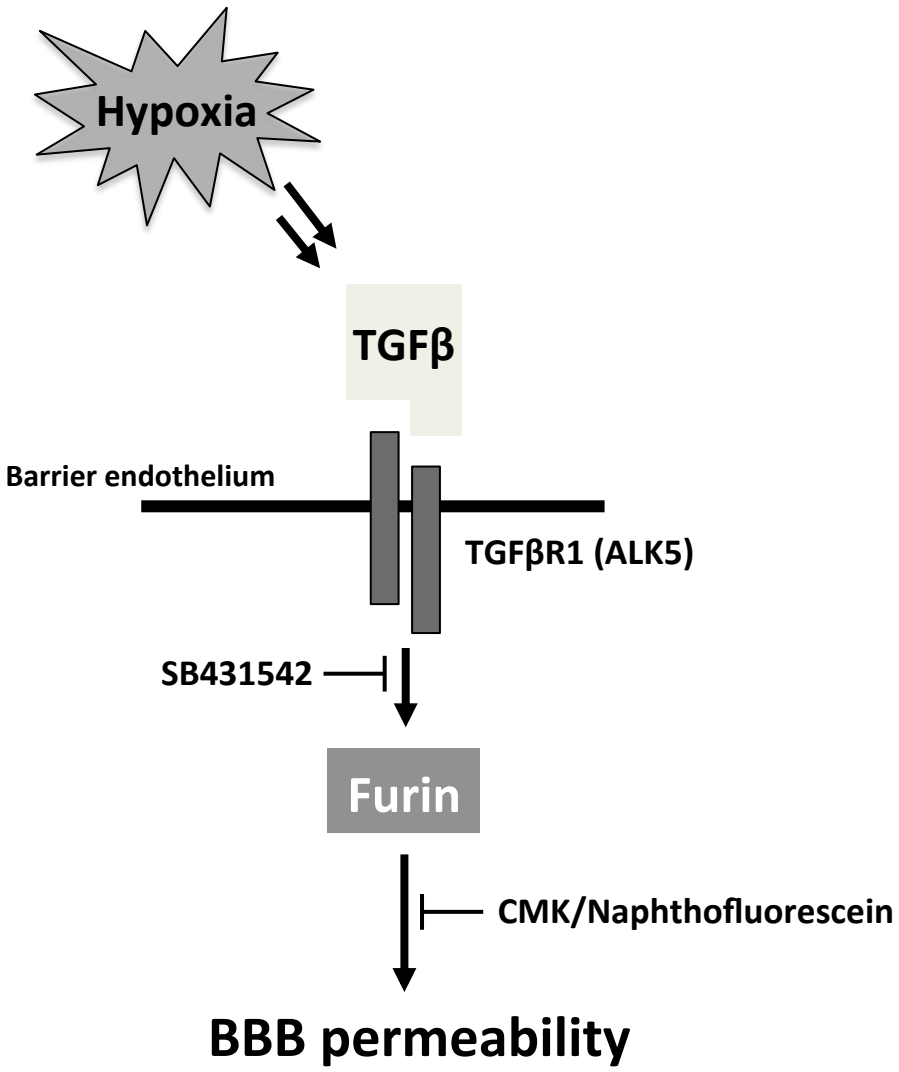
Proposed mechanism of Furin and TGFβ at the BBB



Furin inhibition prevents hypoxic and TGFβ-mediated blood-brain barrier disruption.

Running title: Furin-mediated BBB permeability

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ABSTRACT

Hypoxic blood-brain barrier (BBB) dysfunction is a common feature of CNS diseases however mechanisms underlying barrier disturbance are still largely unknown. This study investigated the role of transforming growth factor β (TGFβ), a cytokine known to induce expression of the proprotein convertase Furin, in hypoxia-mediated barrier compromise. We show that exposure of brain endothelial cells (ECs) to hypoxia (1% O_2) rapidly stimulates their migration. Additional exogenous TGFβ (0.4nM) exposure potentiated this effect and increased Furin expression in a TGFβ type I receptor activin-like kinase 5 (ALK5) - dependent manner (prevented by 10μM SB431542). Furin inhibition prevented hypoxia-induced EC migration and blocked TGFβ-induced potentiation suggesting existence of a feedback loop. TGFβ and Furin were also critical for hypoxia-induced BBB dysfunction. TGFβ treatment aggravated hypoxia-induced BBB permeability but ALK5 or Furin blockade reversed injury-induced permeability changes. Thus during insult Furin compromises endothelial integrity by mediating the effects of TGFβ. Targeting the Furin or ALK5 pathway may offer novel therapeutic strategies for improving BBB stability and CNS function during disease.

Key words: Furin, ALK-5, barrier permeability, cell migration, primary endothelial cells

Introduction

A stable well-functioning blood-brain barrier (BBB) is crucial to maintain and control cerebral homeostasis as well as ensure proper neuronal function. The BBB is formed by specialized brain endothelial cells (EC) that limit paracellular movement of substances to and from the brain parenchyma by their tight junctions (TJ) (1, 2). Pericytes and perivascular astrocytes present at the vascular wall also contribute to barrier maintenance (3). BBB dysfunction allows the influx of damaging molecules, leading to neuronal cell loss and consequently cognitive decline and CNS disease (4). Hypoxia is a key stress factor that induces BBB disturbance and increases vessel permeability through a variety of different mechanisms including EC cytoskeletal reorganization, TJ protein delocalization and altered molecular pathway signaling (5, 6). Hypoxic insults induce the expression of transforming growth factor-β1 (7) and during CNS injury and disease levels of bioactive TGF-β1 increase (7-9). Transforming growth factor betas (TGF-βs) are known multifunctional growth factors that participate in regulation of key events during development, disease, and tissue repair. In the brain TGF-β1, 2 and 3 are broadly expressed with TGF-β1 widely recognized as an injury-related cytokine. Canonical TGF-β signaling is initiated by ligand binding to a high-affinity transmembrane TGF-β type II receptor (TGFβRII) that recruits and phosphorylates the type 1 receptors activin-like kinase 1 and 5 (ALK1, ALK5). Subsequent phosphorylation of the downstream effector proteins Smad2 and Smad3 via ALK5 or Smad 1, 5 and 8 via ALK1 causes formation of a heteromeric complex with Smad4, nuclear translocation and transcriptional regulation (10). At the level of the endothelium TGF-β can signal through both ALK1 and ALK5 and induce different molecular pathways depending on context or dosage (11).

The pleiotropic cytokine TGFβ-1 (henceforth referred to as TGFβ) is involved in many crucial physiological processes such as cell growth, cell differentiation, migration and apoptosis (11) depending on the cell type. For example, whilst the growth factor induced vessel formation in bovine capillary endothelial cells (BCEC), others showed it promoted apoptosis in BCECs and HUVECs and could inhibit the proliferation of bovine heart ECs (12). Targeted deletion of various TGFβ signaling pathway components in mice led to embryonic lethality due to impaired vascular development with leaky vessels (13) and/or angiogenesis defects (14). In correlation dysfunctional TGFβ signaling has been observed in several pathological conditions linked to vascular compromise such as cancer (15), fibrotic disease (16) and atherosclerosis (17). The TGFβ pathway is clearly a major regulator of blood vessel characteristics.

TGFβ has been shown to induce expression of the proprotein convertase Furin in synovial cells and fibroblasts (18). Furin, a calcium-dependent ubiquitously expressed cellular endoprotease (19), is known to process several protein precursors associated with vascular remodeling and cell movement such as vascular endothelial growth factor C (VEGF-C) (20), platelet derived growth factor (PDGF-BB) (21) and membrane-type 1 matrix metalloprotease 1 (MT1-MMP) (22). Very recently it was demonstrated that Furin deficiency in myeloid cells attenuated revascularization after oxygen-induced retinopathy (23). Overall a crucial role for Furin in modulating various vascular endothelia possibly via existence of feedback loops between the enzyme and its substrates has been suggested. In this regard it is important to note that a number of studies using cells of other origin have also convincingly shown Furin to

be a TGFβ converting enzyme (24-26). Whether signaling between these two factors occurs at the BBB and/or modulates barrier dysfunction is unknown.
The aim of this study was to investigate the effect of TGFβ on barrier stability during injury and the potential involvement of Furin in the process. We demonstrate that TGFβ potentiates hypoxia-induced brain EC migration and BBB permeability, and that Furin plays a key role in mediating these effects.

Materials and Methods

Cell culture

The rat brain endothelial cell line RBE4 (27) was used for experiments at passages between 36 and 49 at 80-90% confluency. Cells were maintained on rat-tail collagen coated petri dishes in 1:1 αMEM/Ham's F-10 medium mixture supplemented with 10% FBS, 300µg/mL Geneticin purchased from Gibco® (Thermo Fisher Scientific, MA, USA) and 1ng/mL basic fibroblast growth factor (PeproTech, NJ, USA). Rat-tail collagen was isolated as previously described (28) and diluted to 250µg/mL in 70% Ethanol.

Primary endothelial cell isolation

Primary rat brain endothelial cells (primary ECs) were isolated from 8-10 week old male Wistar rats according to Coisne et al. (29), with slight modifications. Cortices were homogenized after removal of meninges in a Dounce homogenizer. Microvessels were isolated by adding an equal volume of 30% dextran solution to the homogenate and subsequent centrifugation at 3000g for 25 min at 4°C, then filtered through a 60µm nylon mesh to remove larger vessels. After digestion in HBSS buffer supplemented with 10mM HEPES, 0.1% BSA, 2mg/mL collagenase-dispase, 10µg/mL DNase I and 147ng/mL TLCK, for 45min at 37°C, microvessels were re-suspended in endothelial media (DMEM Glutamax II supplemented with 10% calf serum, BME amino acids, vitamin solution, 2mM L-glutamine, 1ng/mL bFGF, 59µg/mL gentamycin sulfate). After plating on collagen IV coated culture dishes pericyte contamination was prevented by exposing cultures to 3µg/mL puromycin-containing media for 24h. Cells were used in experiments on day 5 without passaging.

Hypoxic and substance exposures

Hypoxic exposures were performed in a hypoxic glove box chamber at 37°C, 5% CO_2 and 1% O_2 (InVivo$_2$ 400, Ruskinn Technologies, Pencoed UK). An internal O_2 sensor monitored O_2 concentrations constantly. The following substances were added exogenously to the cell media: 0.4nM human TGFβ-1 (PeproTech) dissolved in 10mM citric acid, 10µM ALK5 inhibitor SB431542 (Tocris, Bristol, UK) in DMSO, 20µM Naphthofluorescein (Furin inhibitor, Santa Cruz, TX, USA) in DMSO, 20µM Decanoyl-Arg-Val-Lys-Arg-chloromethylketon (CMK, Furin inhibitor, Santa Cruz) in H_2O. Cells were pretreated with inhibitors for 1h prior to hypoxic and TGFβ exposures.

Cell viability

Viability of confluent cells was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT solution (Sigma-Aldrich, MO, USA) was added to the media (final concentration 0.5mg/mL) and incubated for 1h at 37°C. The

media was removed and formazan crystals dissolved in DMSO. Optical density was measured at 570nm on a spectrophotometer (Thermo Labsystems, Multiskan RC Model 351) with reference filter at 670nm.

Cell migration

RBE4 cells were grown in 6-well plates and a scratch was introduced vertically with a 200µL pipette tip. Three pictures were taken per well before and after the exposures with an inverted microscope coupled to an 8-bit CCD camera (Axiocam HR, Carl Zeiss, Switzerland). Image analysis using ImageJ software (NIH, USA) was used to determine scratch width. Mean migration (µm/h) was calculated by evaluating the difference in gap size before and after the exposure.

Permeability assay

ECs were grown to confluency on 0.4µm Transwells (Corning Incorporated, NY, USA). After exposures fresh medium containing 1mg/mL Lucifer yellow CH, lithium salt (Life technologies, OR, USA) was added to the upper compartment. At 9, 15, 30, 45 and 60 min aliquots were taken from the bottom compartment and tracer flux was determined with a fluorescence plate reader (FLx800, Biotek Instruments, Winooski, VT). A clearance slope established from the measurements obtained at the different time points was used to calculate the permeability coefficient (Pe) (30).

Western blotting

Cells were lysed in whole cell lysis buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, 1% NP-40) supplemented with protease inhibitor cocktail (Calbiochem, Darmstadt, Germany), 1mM sodium orthovanadate and 0.5mM phenylmethansulfonyl fluoride. Protein concentrations were determined with a Pierce BCA protein assay (Thermo Fischer Scientific). Proteins (50µg) were separated by SDS-PAGE then transferred to nitrocellulose membranes and blocked with 5% non-fat dried milk or 5% BSA in TBS. After incubation in primary antibodies over night at 4°C membranes were washed then incubated with the corresponding HRP-conjugated secondary antibody. Bands were visualized with a luminescent image analyzer (Fujifilm, LAS-3000, Switzerland) and quantification was performed with ImageJ software (NIH, USA) using β-actin as loading control. The following antibodies were used: anti-Furin (SC-20801, Santa Cruz), anti-HIF-1α (NB100-479 W-1, NovusBio), anti-pSmad2 (138D4, Cell Signaling), anti-Smad2 (D43B4, Cell Signaling), anti-Smad1/5 (D5B10, Cell Signaling), anti-Smad1 (D59D7, Cell Signaling) and β-actin (A5441, Sigma-Aldrich).

Quantitative RT-PCR

RNA was isolated from cells using TRIzol® reagent (Thermo Fisher). Total RNA (1 µg per sample) was reverse transcribed with oligo-dT primers using the ImProm-II ReverseTranscriptase kit (Promega, Switzerland) according to the manufacturers protocol. Real-time quantitative PCR (qPCR) was performed with an ABI 7500 Fast Real-Time PCR System using Power SYBR® Green Master Mix (Applied Biosystems, Switzerland). The following primers were employed: Furin 5'-CAG AAG CAT GGC TTC CAC AAC-3' and 5'-TGT CAC TGC TCT GTG CCA GAA-3' and β-actin 5'-CTG GCT CCT AGC ACC ATG AAG-3' and 5'-GCC ACC GAT CCA CAC AGA GT-3'. Serial cDNA dilution series were used to establish optimal primer conditions resulting in >90% efficiency. To exclude primer dimer and off-target amplifications melting curves were performed and confirmed by gel

electrophoresis. Data was normalized to β-actin and fold changes calculated using the ΔΔCt method.

Statistical Analysis

Statistical Analyses were performed with Prism GraphPad 6. Results are shown as mean values ± standard deviation with a minimum of 3 independent experiments. Statistical significance was determined by two-way ANOVA when comparing more than two different groups with different exposures or unpaired Student's t-test with homoscedasticity. A p value less than 0.05 was considered significant.

RESULTS

TGFβ promotes EC migration

Till now the role of TGFβ signaling has been mainly studied in peripheral ECs whereas its effects on the brain endothelium remain largely debated. In the first instance, we aimed to establish how increased TGFβ levels impact brain EC quiescence during hypoxic injury using a classic scratch migration assay and the rat brain microvascular endothelial (RBE4) cell line. Notably, our studies have shown this cell line exhibits the same responses as primary cells but with increased sensitivity to oxygen deprivation (6). Scratch assays were performed during 6h normoxic and hypoxic exposure with exogenous TGFβ and ALK5 inhibitor treatment (Fig. 1A&B). Quantification showed no significant changes under normoxic conditions with any of the treatments (Fig. 1C). In contrast hypoxia significantly increased EC migration and TGFβ potentiated this effect. Notably, both hypoxic and TGFβ-mediated migration were completely blocked when ECs were pretreated with the TGFβ receptor I inhibitor (SB431542). Thus TGFβ potentiates hypoxia-mediated cell migration via a TGFβR1 dependent mechanism.

Hypoxia and TGFβ modulate Furin protein and expression and activation

Since existence of a feedback loop between TGFβ and Furin has been implicated in various other (non-vascular) cells (24-26), we asked if a similar mechanism could underlie the movement we observed. As Furin mRNA levels were unaffected in our treatment conditions (Supp. Fig. 1) we evaluated whether Furin activation correlated with the TGFβ-induced migratory effects. Hypoxic HIF-1α stabilization confirmed the endogenous endothelial cell response was as expected and unaffected by TGFβ treatment (Fig. 2A). Corresponding Furin immunoblots showed two prominent bands corresponding to the inactive precursor form at 96kDa and the mature active form at 90kDa (Fig. 2A). Quantitative analysis of the individual bands revealed a significant decrease of the Furin precursor under hypoxia. Notably, TGFβ had no impact on normoxic precursor levels but induced hypoxic precursor levels by 50% to reach the baseline normoxic levels (Fig. 2B). Mature Furin levels remained unchanged by hypoxia compared to the normoxic baseline (Fig. 2C) but significantly increased during hypoxic TGFβ exposure, with a trend to also increase in normoxia. This data suggests that during stress (hypoxic) conditions high TGFβ levels might potentiate EC Furin activation by elevating precursor levels and inducing protein maturation.

Furin activation is dependent on ALK5 signaling

In ECs, canonical binding of TGFβ to ALK5 causes downstream activation and phosphorylation of Smad2 resulting in stimulation of angiogenesis (31), whereas signaling via ALK1 induces phosphorylation of Smad1, 5 and 8 and subsequent endothelial quiescence (32). Exposure of brain ECs to TGFβ but not hypoxia increased phosphorylated Smad2 levels whereas the opposite effect was observed on phospho-Smad1/5 levels (Supp. Fig. 2&3). In line with suppressing cell migration (Fig. 1), ALK5 blockade also prevented TGFβ-mediated Smad2 phosphorylation but not the hypoxia-induced Smad1/5 activation. As Furin levels were also strongly potentiated by both hypoxia and TGFβ (Fig. 2) we asked if ALK5 blockade would suppress these effects (Fig. 3). Indeed immunoblotting showed SB431542 abrogated expression of the Furin precursor and mature forms by more than 50% during hypoxia and TGFβ exposure (Fig. 3). Interestingly, this pattern was also observed during normoxia (Supp. Fig. 4) suggesting Furin activity is highly dependent on ALK5 activation in both injury and physiological conditions.

Furin inhibition prevents EC migration

We next evaluated if Furin is the mediator of hypoxia- and TGFβ-induced EC migration. A scratch assay was performed over 6h hypoxia as previously in the presence and absence of TGFβ and the Furin inhibitors Naphthofluorescein (33) and CMK. Clear differences were apparent after visual inspection of the samples (Fig. 4A). Quantification showed hypoxia-induced cell migration was significantly potentiated when combined with exogenous TGFβ as expected however treating cells with Furin inhibitors completely abrogated this response even in the presence of TGFβ (Fig. 4B). Notably, both inhibitors also blocked normoxic EC migration by up to 60% (Supp. Fig. 5). These observations highlight that TGFβ-induced EC migration is mediated by Furin and again underline an important physiological role for Furin.

TGFβ potentiates hypoxia-mediated EC permeability

To understand whether injury-increased TGFβ levels contribute to altered barrier integrity we performed permeability assays on primary microvascular ECs isolated from adult rat brain and RBE4 (data not shown) in presence and absence of exogenous TGFβ (Fig. 5). Experiments were performed for 24h (Fig. 5A) and 48h (Fig. 5B) under normoxic and hypoxic conditions. Hypoxia significantly increased permeability of EC monolayers as frequently observed in many studies including our own (3). Exposure to TGFβ further potentiated hypoxia-induced barrier dysfunction although no effects were observed under normoxia (Fig. 5A). Pretreatment with SB431542 completely abrogated TGFβ and hypoxic driven effects to baseline normoxic levels. Indeed at both time points a similar outcome was seen although the effects at 48h were clearly more enhanced (Fig. 5A&B). Thus sustained injury-induced activation of the endothelial TGFβ-ALK5 pathway culminates in barrier disturbance.

Furin inhibition prevents hypoxia-induced barrier permeability

Finally, due to the observed feedback between the proteins, we investigated if Furin inhibition would also suppress the barrier changes induced by hypoxic and/or TGFβ exposure. Again we employed the transwell permeability assay using primary rat ECs (Fig. 6A) and RBE4 (data not shown). Blocking Furin activation with CMK was highly efficient at preventing hypoxia-mediated barrier disruption after 48h. Similar to observations in the migration assay, the CMK inhibitor resulted in a tighter monolayer than observed under normoxic conditions (Fig. 6A). Surprisingly however

the combination of CMK and exogenous TGFβ dramatically increased EC barrier permeability by more than 5 fold. This led us to use MTT assays to assess if the observed permeability changes were due to compromised cell viability. Data showed that when combined with hypoxia TGFβ treatment already compromised cell function up to 20%. Whilst CMK alone had no effect, the combination treatment reduced mitochondrial activity by 50% (Fig. 6B). Thus Furin blockade in the presence of elevated TGFβ reduces cell viability and strongly impairs BBB functionality.

DISCUSSION

Maintaining BBB integrity is crucial for CNS homeostasis. Hypoxia and ischemia are well known to compromise barrier stability via TJ and extracellular matrix disruption leading to permeability changes (5). Such alterations ultimately contribute to disease progression (4). This study shows that both hypoxia and exogenous TGFβ promote cell migration and barrier dysfunction via activation of the proprotein convertase Furin. Blocking Furin abrogates hypoxia and TGFβ-induced endothelial migration and permeability indicating a potential future target for vascular-related interventions.

Stimulatory effects of TGFβ on cell migration have been reported in intestinal (34) and renal epithelial cells (35), renal microvascular EC (36) and cancer cells (37) under normoxic conditions although high doses of TGF-β may inhibit non-brain EC migration (11, 36). In contrast TGFβ did not induce migration of brain ECs in normoxia but only during hypoxic insult in correlation with a study performed on fibroblasts (38). Our observations thus highlight that TGFβ signaling activates angiogenic processes in brain EC only during injury conditions, a poignant difference between peripheral cell types and brain EC.

TGFβ induces the expression of Furin, a proprotein convertase whose up regulation consistently correlates with tumor cell activation and invasiveness (39). In synovial cells (40) and bovine aortic endothelial cells (41) TGFβ was shown to increase Furin transcription. Despite no clear effects on mRNA levels, TGFβ treatment clearly induced Furin protein levels in brain EC. Endothelial TGFβ can signal through two different TGFβ type I receptors, ALK5 and ALK1, which either promote angiogenesis or induce vascular quiescence respectively (11, 32). TGFβ signaling through ALK5 activates Smad2 and 3 whilst signaling through ALK1 activates Smad1, 5 and 8 (8, 42). In brain ECs differential Smad signaling was clearly stimuli-related with Smad1/5 phosphorylation being hypoxia-dependent and Smad2 activation being TGFβ-dependent. It seems likely that a balance between activation of ALK1 versus ALK5 determines whether ECs remain quiescent or initiate an angiogenic response, an observation previously described by Goumans et al in 2002 (11). In HUVEC and bovine aortic EC ALK5 majorly contributed to angiogenic processes (31, 43). Similarly ALK5 blockade prevented TGFβ-induced activation of Smad2 (without affecting pSmad1/5 levels) and suppressed brain EC migration in agreement with those findings. Notably, ALK5 activation was also linked to BBB dysfunction in mouse brain endothelial cell (bEnd.3) monolayers wherein TGFβ-induced permeability, via down regulation of the TJ protein Claudin-5, was mediated by Smad3 (44). Overall our data supports the notion that suppressing ALK5 activation

296 could be key for maintaining BBB function. Our data also suggests Furin modulation
297 is strongly dependent on TGFβ/ALK5 signaling and mediated by Smad2 in brain EC
298 in line with other work (18, 40, 45). Whether Smad2 directly or indirectly regulates
299 the effects of TGFβ on Furin levels is currently under investigation.
300 A curious observation from this work is that TGFβ induced mature Furin levels under
301 both normoxic and hypoxic conditions but enhanced migration only occurred during
302 hypoxia. Since Furin is a bona fide HIF-1 target (46) it seems plausible that the HIF-1
303 pathway could somehow contribute to this effect. HIF-1 targeted knockdown studies
304 will further clarify this involvement.
305
306 Convertase enzymes exist in an immature preform with a prosegment still attached.
307 Several studies have demonstrated the use of convertase prosegments as inhibitors of
308 their parent enzymes *in vitro* and *ex vivo* (47, 48). During the maturation of Furin, the
309 prosegment acts as a chaperone in the auto-activation pathway as well as a potent
310 auto-inhibitor (49). Indeed profurin overexpression in vascular smooth muscle cells
311 decreased endogenous Furin protein levels and reduced cell proliferation and
312 migration (50). Further hepatic overexpression of the Furin prosegment reduced
313 atherosclerosis progression and decreased vascular remodeling after injury *in vivo*
314 (50). In analogy we observed downregulation of the Furin precursor during hypoxia
315 indicating that potential inhibition of maturation is lifted, resulting in increased Furin
316 activity in line with our migration assay results. This data supports the conclusion that
317 increased Furin activity is ultimately responsible for hypoxic EC migration especially
318 since Furin inhibition not only prevented hypoxic EC migration but also abrogated
319 TGFβ-induced effects. In agreement Furin inhibition also attenuated TGFβ-enhanced
320 migration of cardiac fibroblasts (51), reduced invasiveness/migration and
321 tumorigenicity of human cancer cells *in vivo* (52) and *in vitro* (47). Hence similar
322 mechanisms underlie brain EC migration and likely contribute to increased barrier
323 permeability during injury conditions. Although not elucidated here, a possible
324 mechanism of Furin-mediated cell movement is likely linked to processing of matrix
325 metalloproteinases (MMPs). Synthesized as inactive propeptides, MMPs undergo
326 endoproteolytic cleavage to attain their active forms (53) resulting in extracellular
327 matrix degradation and barrier disruption (54). Accordingly hypoxia is known to
328 increase Furin-mediated MT1-MMP processing (22, 46). Since Furin inhibition in
329 human cancer cells and cardiac fibroblasts decreased processing and activation of
330 MT1-MMP/MMP-2 (51, 55) it is very tempting to speculate that TGFβ-induced brain
331 EC migration is driven by Furin-mediated activation of MMPs.
332
333 At the BBB, a TGFβ-induced migratory (activated) EC phenotype implies barrier
334 compromise as cytoskeletal alterations lead to a consequential loss of tight junction
335 organization. Indeed TGFβ induced brain EC migration and permeability and
336 aggravated injury-induced barrier compromise via an ALK5-dependent mechanism in
337 contrast with a study showing that activation of ALK5 by TGFβ inhibits migration in
338 non-brain ECs (13). The unique qualities of BBB ECs including lack of fenestrations,
339 more extensive tight junctions, and reduced pinocytic vesicular transport is clearly
340 significant (6). Although decreased viability of brain EC observed during TGFβ
341 treatment was unexpected our data further underscores that negative EC responses to
342 the growth factor can be reversed by inhibiting either ALK5 or Furin activity. Indeed
343 blocking of either abrogated hypoxic barrier disruption agreeing with the notion that
344 these pathways are tightly entwined and induce a migratory/activated phenotype. It is
345 now very evident that Furin activation also plays an important role in injury-induced

BBB disturbance. However an intriguing and unexpected finding, given Furin inhibition prevented short-term hypoxic and TGFβ-induced migration, was that combined prolonged TGFβ treatment and Furin blockade caused more disruption than TGFβ stimulus alone in correlation with dramatically compromised viability. The reason behind this is unclear but disruptive and negative time-dependent effects of sustained TGFβ signaling at the BBB and potential cofounding effects of modulating multiple downstream pathways must be better understood.

Overall our data suggests both hypoxia- and TGFβ-mediated EC permeability can be prevented by blocking Furin and/or ALK5 activation. Notably, a large source of native active TGFβ that can be released upon different injury stimuli *in vivo* lies within the perivascular cells and/or the extracellular matrix. Thus further studies using more complex BBB models such as co-culture of ECs with perivascular cells will be needed to provide further insight into the highly complex molecular mechanisms and cellular cross-talk that take place during injury in vivo. In summary, controlling Furin or ALK5 activation at the BBB could offer a novel therapeutic option for improving barrier functionality and CNS function during disease.

FIGURE LEGENDS

Fig. 1: TGFβ promotes EC migration

Representative micrographs of scratched confluent RBE4 monolayers exposed to 0.4nM TGFβ, the ALK5 inhibitor SB431542 (10μM) or a combination of both prior to Nx (A) or Hx (B) incubation for 6h. Scale bar=100μm. Images were quantified and mean migration in μm/h is presented (C). Mean ± SD (n=3), 2way ANOVA, **p<0.01 compared to Nx UNT, #p<0.05 ##p<0.01 ###p<0.001 compared to Hx TGFβ.

Fig. 2: Both hypoxia and TGFβ modulate Furin

(A) Representative Western blot of Furin precursor (96KDa) and mature (90KDa) forms and HIF-1α stabilisation at 6h hypoxia with and without TGFβ treatment. Blot quantification and graphical representation shows changes of the Furin forms under hypoxia and TGFβ treatment (B&C). β-actin was used as loading control. Data are expressed as mean ± SD of at least n=3 independent experiments. Students t-test and 2way ANOVA, *p<0.05 compared to Nx UNT, §p<0.05 compared to Hx UNT.

Fig. 3: Furin activation is dependent on TGFβ receptor-1 ALK5 signaling.

(A) Representative blot showing Furin protein expression after TGFβ or SB431542 treatment. Quantitative graphs show comparative levels of both precursor (B) and mature (C) Furin. Mean ± SD (n=3), Students t-test, §p<0.05, §§p<0.01 compared to Hx UNT, ###p<0.001 to Hx TGFβ.

Fig. 4: Furin inhibition prevents EC migration

Representative micrographs of scratched RBE4 monolayers incubated with TGFβ, the Furin inhibitors Naphthofluorescein (Naph, 20μM) or CMK (20μM) or a combination of both prior to Hx exposure for 6h (A). Scale bar =100μm. Quantified data (B) shows changes in EC migration under all conditions. Mean ± SD of at least n=3 independent experiments. 2way ANOVA, ***p<0.001 compared to Nx UNT, ###p<0.01, ##p<0.01 compared to Hx TGFβ, §§p<0.01 to Hx UNT.

Fig. 5: TGFβ potentiates hypoxia-mediated EC permeability via ALK5

Permeability assay was performed on primary rat endothelial cells grown to confluency on transwells then exposed to 0.4nM TGFβ or 10μM SB431542 during 24h (A) and 48h (B) hypoxia. Dashed line indicates normoxic baseline measurements. 2way ANOVA and Students t-test, §§p<0.01, §§§p<0.001 compared to Hx CTRL, ##p<0.01 compared to Hx TGFβ treatment, Students t-test.

Fig. 6: Furin inhibition prevents hypoxia-induced barrier permeability

Primary rat endothelial cells were grown to confluency on transwells or culture dishes before hypoxic exposure to 0.4nM TGFβ, CMK (20μM) or CMK combined with TGFβ for 48h. Permeability assays (A) and MTT assays (B) were subsequently performed. Mean ± SD (n=4), 2way ANOVA and Students t-test, **p<0.01, ****p<0.0001 compared to Normoxia, ##p<0.01 to Hx TGFβ, §§p<0.01, §§§p<0.001 to Hx UNT.

Supplementary Figure 1: Furin mRNA levels

Quantitative real time PCR shows changes in Furin mRNA levels after treatment with 0.4nM TGFβ, 10μM ALK5 inhibitor SB431542 or a combination of both prior to 6h

Nx or Hx exposure. Fold changes were calculated with the ΔΔCt method and normalized to Nx UNT. Mean ± SD (n=4), 2way ANOVA.

Supplementary Figure 2: TGFβ activates Smad2 via ALK5

Representative Western blot (A) and quantification (B) of total and pSmad2 levels after exposure to 0.4nM TGFβ, the ALK5 inhibitor (10μM) or a combination of both prior to Nx or Hx incubation for 6h. Mean ± SD (n=4), Students t-test, **p<0.01, ***p<0.001 compared to Nx UNT, #p<0.05 compared to Hx TGFβ, °°p<0.01 compared to Nx TGFβ.

Supplementary Figure 3: Smad1/5 signaling is unaffected by ALK5 blockade

Representative Western blot (A) and quantification (B) of p-Smad1/5 and Smad1 (60kD) protein levels after 6h normoxic or hypoxic exposure with TGFβ, ALK5 inhibition or a combination of both (A). Mean ± SD (n=3), Students t-test, *p<0.05.

Supplementary Figure 4: Furin and ALK5 signaling under normoxia

Representative immunoblot of Furin protein levels after TGFβ or SB431542 treatment under normoxic conditions (A). Changes in Furin precursor (B) and mature (C) levels were subsequently quantified. Mean ± SD (n=4), Students t-test,*p<0.05, **p<0.01 to Nx UNT.

Supplementary Figure 5: Furin inhibition blocks normoxic EC migration

Mean migration (μm/hr) of normoxic RBE4 cells after exposure to TGFβ (0.4nM) or a combination of TGFβ and the Furin inhibitors Naphthofluorescein (20μM) and CMK (10μM). Mean ± SD (n=3-5), Students t-test,*p<0.05, **p<0.01 to Nx UNT.

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Figure 1: TGFβ promotes EC migration

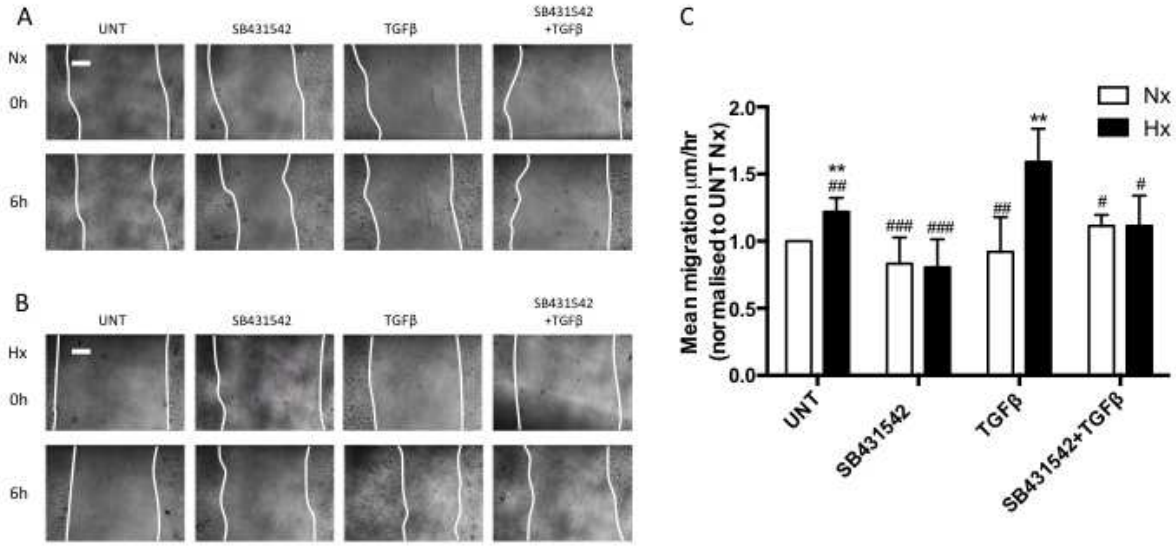


Figure 2: Both hypoxia and TGFβ modulate Furin

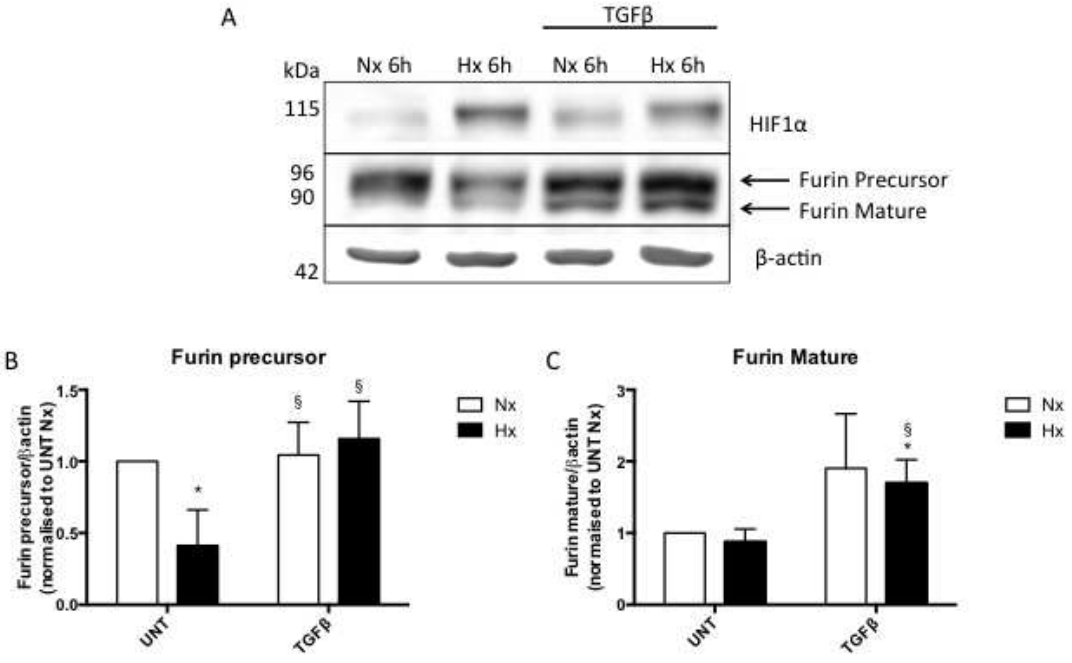


Figure 3: Furin activation is dependent on ALK5 signaling

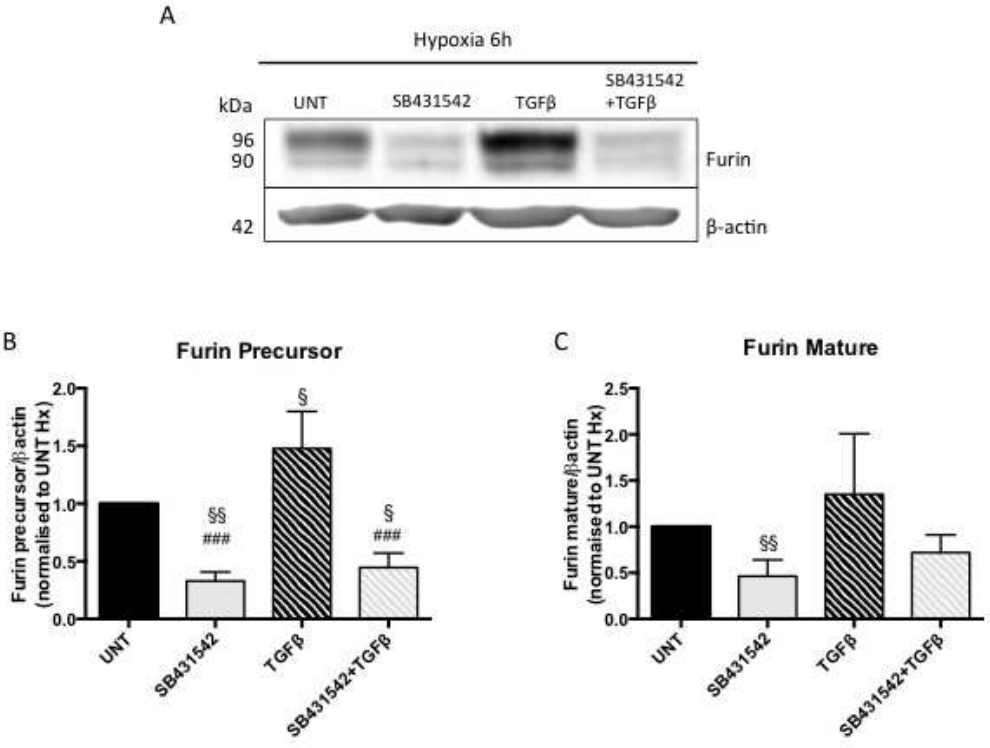


Figure 4: Furin inhibition prevents EC migration

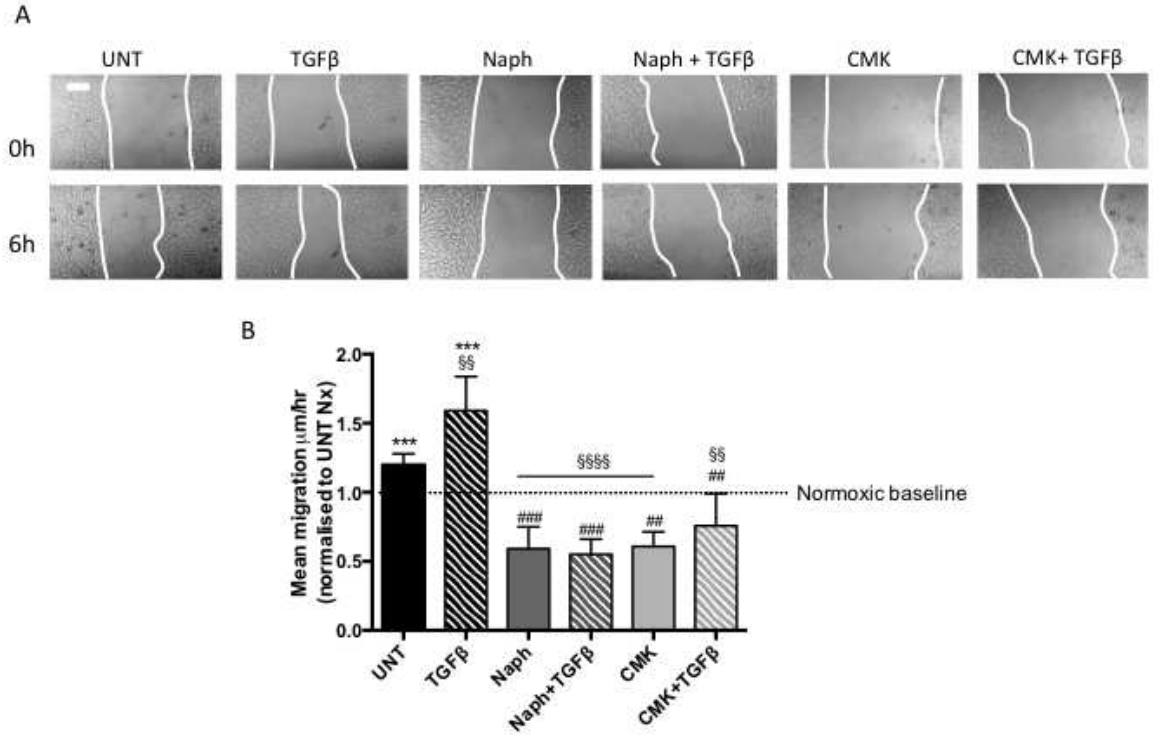


Figure 5: TGFβ potentiates hypoxia-mediated EC permeability via ALK5

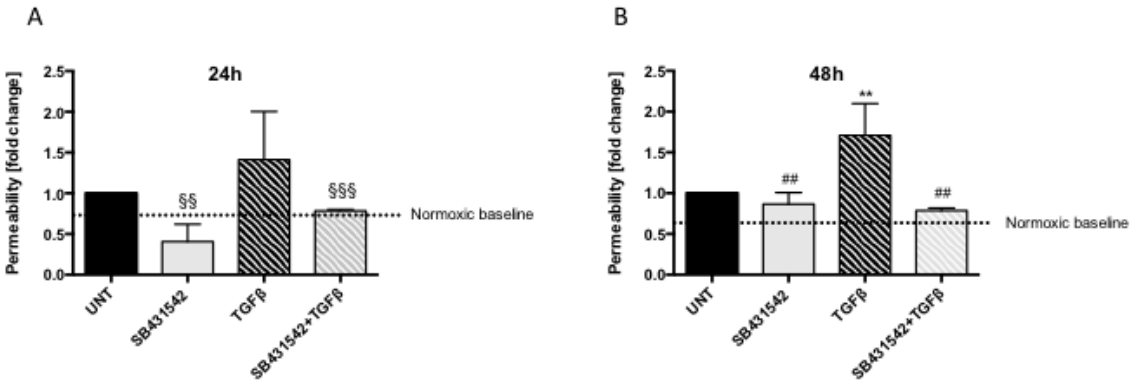
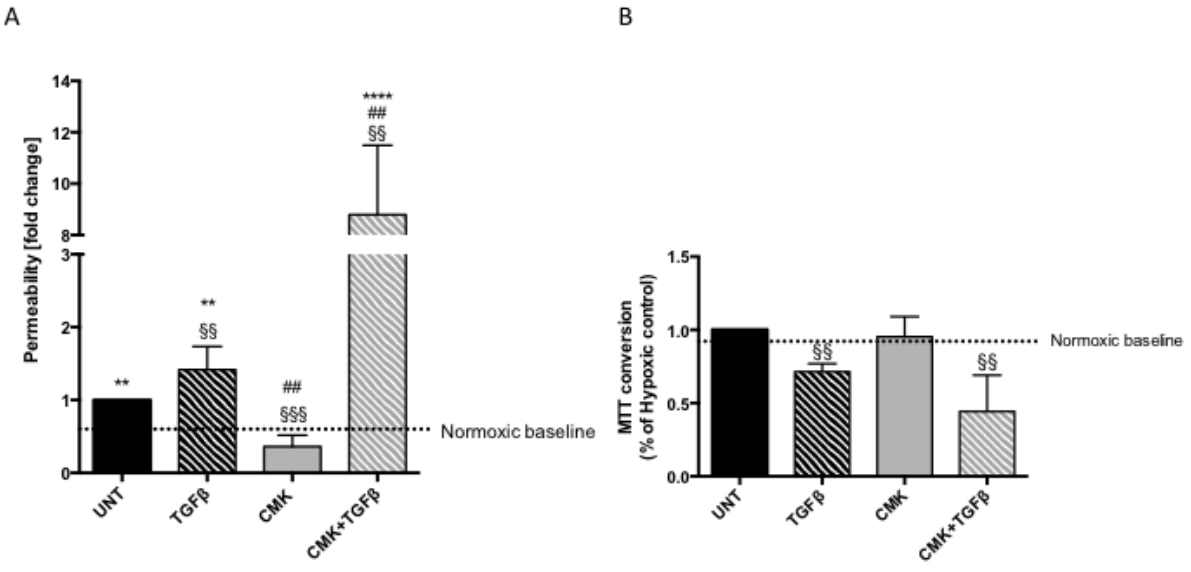


Figure 6: Furin inhibition prevents hypoxia-induced barrier permeability



AUTHOR CONFLICT OF INTEREST STATEMENT (for ECR-19-511)

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfy the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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